Reference: *Biol. Bull.* **164:** 28–40. (February, 1983)

ANTIBACTERIAL ACTIVITY OF THE COELOMIC FLUID OF THE POLYCHAETE, *GLYCERA DIBRANCHIATA*. I. THE KINETICS OF THE BACTERICIDAL REACTION

BENJAMIN M. CHAIN AND ROBERT S. ANDERSON

Sloan-Kettering Institute for Cancer Research, Donald S. Walker Laboratory, 145 Boston Post Road, Rye, New York 10580

Abstract

The kinetics of the bactericidal action of cell-free coelomic fluid from *Glycera dibranchiata* on a Gram-negative bacterium, *Serratia marcescens*, were studied using both a turbidometric growth assay and a direct plating assay. The bactericidal reaction consisted of two stages involving an initial rapid binding to the bacterial surface, followed by a slower killing reaction. The first stage required the presence of divalent cations and was slightly temperature-dependent. In contrast, the rate of the second stage was accelerated at temperatures normally optimal for multiplication of the bacteria. Kinetic studies and an analysis of the dose-response curve at low concentrations indicated that three or four hits by the bactericidal factor are required to kill a bacterium.

Introduction

The coelomic fluid of the marine worm, *Glycera dibranchiata*, has been shown to possess potent bactericidal properties against several Gram-negative bacteria. Preliminary studies showed that the bactericidal activity resides in a heat-labile protein with a molecular weight of >100,000 (Anderson and Chain, 1982). *Glycera* antibacterial factor (GAF) presumably acts as part of the natural humoral immunity system of the worm and defines a new class of animal-derived antibacterial molecules. Two major questions of interest are the mode of action and biochemical structure of GAF. This paper will describe kinetic experiments which are aimed at elucidating the first of these questions, the accompanying paper will address the second. Ideally, it would have been more satisfactory to purify and characterize GAF before undertaking kinetic studies, but this proved technically difficult.

The results of these kinetic studies suggest a two-step model for GAF bactericidal action involving an initial binding to the bacterial surface and a subsequent "killing reaction."

Materials and Methods

Glycera dibranchiata (blood worms) were obtained from the Maine Bait Co. and kept in artificial sea water aquaria at 12°C. Methods for bleeding the worms and preparing cell-free sterile coelomic fluid (CF) were described previously (Anderson and Chain, 1982).

Two types of assay of bactericidal activity, using overnight cultures of *Serratia marcescens* as the test organism, were adapted for use with *Glycera* coelomic fluid. The first was based on the turbidometric assay of Muschel and Treffers (1956a) for

Received 10 May 1982; accepted 8 October 1982.

measuring complement activity. A known volume of coelomic fluid was diluted in artificial sea water (ASW) to give a total volume of 0.5 ml. GAF activity was greater in ASW than in the phosphate buffer system employed in earlier studies (Anderson and Chain, 1982). A bacterial suspension of *S. marcescens* ($\sim 6 \times 10^5$ bacteria/0.1 ml) was added to the coelomic fluid. After gentle mixing, the bacteria-coelomic fluid mixture was incubated, routinely at room temperature, for a period of 1–30 minutes. The effects of various incubation temperatures were also studied. The incubation was terminated by dilution with 4 ml cold tryptic soy broth (TSB) and the cultures maintained at 1–4°C until all portions of the experiment were complete. The bacteria were then grown up at 37°C until control tubes (without coelomic fluid) reached $O.D._{570} \cong 0.25$. The relative optical densities were a measure of the number of bacteria surviving exposure to coelomic fluid. For quantitative studies, a standard curve was used to convert the relative O.D.s to the number of bacteria surviving exposure to GAF, present at the start of the growth period (Anderson and Chain, 1982).

The other assay used was a direct plating assay. Coelomic fluid and bacteria were allowed to react at room temperature, or at 37°C, for periods of 10 minutes to 2 hours. The suspension was diluted (1:100 in 0.9% saline) to stop the reaction, mixed thoroughly, and 100 μl aliquots were plated in 10 ml tryptic soy agar. The plate counts obtained were a direct measure of the number of bacteria surviving exposure to the coelomic fluid.

Artificial sea water was made up as a 38 g/l solution of Instant Ocean (Aquarium Systems, Inc.). The concentrations of the major ionic components were Na^+, 443 mM, K^+, 9.5 mM, Ca^{2+}, 9.4 mM, Mg^{2+}, 50 mM, Cl^-, 518 mM, and SO_4^{2-} 26 mM. All other chemicals were obtained from the Sigma Chemical Company. Dialysis experiments were done at 1°C using Spectrapor 2 membrane tubing (M. W. cutoff, 12,000–14,000).

Results

The nature of the assay

In our initial studies there was an apparent lack of agreement between the data obtained from the two assays described above; these discrepancies are shown in Figure 1. In the turbidometric assay, low concentrations of CF produced a stimulatory effect on bacterial growth not seen in the plate assay. This was probably due to the stimulatory effect of certain metal ions present in CF on the subsequent growth of the bacteria. When the reaction was carried out in ASW rather than in sodium phosphate buffer (0.1 M, pH 7.5), no stimulatory effect was observed (Fig. 2). In addition, the time required to run the assay was considerably shortened. The action of divalent cations in shortening the lag phase of bacterial growth has been well documented (*e.g.*, Guirard and Snell, 1962).

The total bactericidal activity of CF was also greatly enhanced in ASW (Fig. 2). The CF volume required for a 50% inhibition of optical density was reduced from 18.6 ± 1.9 μl ($\bar{X}$ ± SEM, N = 5) to 4.6 ± 0.5 μl ($\bar{X}$ ± SEM, N = 11). The ionic requirements of GAF were studied further in dialysis experiments. Coelomic fluid was dialyzed overnight against a large volume of Tris buffer (0.1 *M*, pH 8) containing 0.01 *M* $CaCl_2$ shown to be required to prevent irreversible loss of activity. The dialyzed CF was assayed for bactericidal action in media of different composition, and the activity (the volume of CF required to obtain a 50% reduction in optical density, relative to controls) was determined (Table I). Nearly total original activity of dialyzed CF was restored when tested in ASW rather than in dialysis buffer.

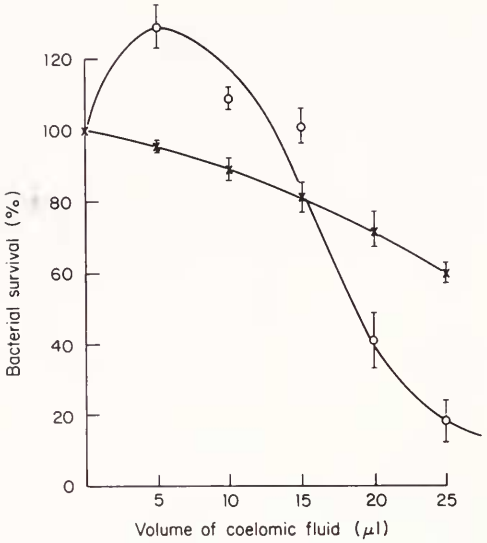

FIGURE 1. A comparison of the turbidometric assays (○) and plate assays (X) of bactericidal activity; mean ± S.E.M. (N = 3). Incubations were for 30 min at room temperature in sodium phosphate buffer (pH 7.5, 0.1 *M*).

Maximal activity was observed when magnesium and sodium ions in concentrations approximating those found in sea water, were added to the dialysis buffer.

Another major discrepancy between the two assays was the much greater apparent activity recorded by the turbidometric assay, in comparison to the plate assay (Fig. 1). In the hope of clarifying this anomaly, the changes in bacterial numbers

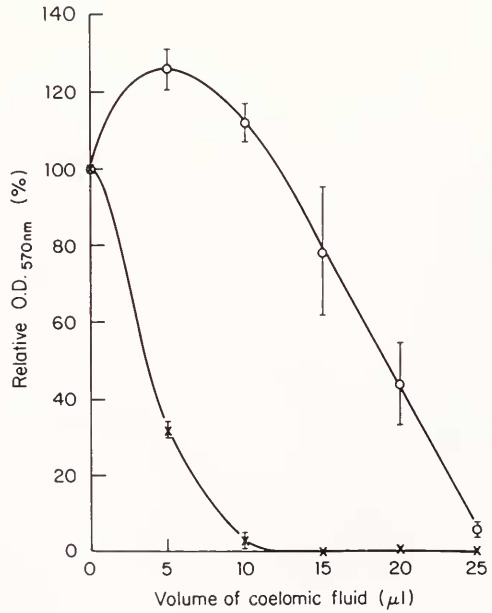

FIGURE 2. A comparison of the turbidometric bactericidal assay in sodium phosphate buffer (○) and in artificial sea water (X); mean ± S.D. (N = 3). Incubations were for 30 min at room temperature.

TABLE I

The influence of assay medium on bactericidal activity

Treatment of CF	Assay medium	CF Volume (μl) required for 50% reduction in O.D.
Undialyzed	Artificial sea water	4.8
Dialyzed	Artificial sea water	5.5
Dialyzed	DB (Dialysis buffer: 0.01 *M* Tris, pH 8, containing 0.01 *M* $CaCl_2$)	14.5
Dialyzed	0.5 *M* $MgCl_2$ in DB	12.0
Dialyzed	0.5 *M* NaCl in DB	11.5
Dialzyed	0.1 *M* NaCl inDB	17.0
Dialyzed	0.5 *M* $MgCl_2$ + 0.5 *M* NaCl in DB	5.5

Coelomic fluid was dialyzed overnight against a 0.01 *M* $CaCl_2$ + 0.01 *M* Tris buffer (pH 8.0). Dialysates (0–20 μl) were assayed (in triplicate) using the turbidometric assay and 50% activity values calculated from the dose-response curves obtained.

during the early part of the amplification (bacterial growth) period of the turbidometric assay (*i.e.*, from the time the broth was added to the bacteria until an optical density could be recorded) were measured directly by plating successive samples (Fig. 3). The results were unexpected. The numbers of viable bacteria were almost unchanged throughout the "reaction period" at room temperature and subsequently, for as long as the tubes were maintained at 1–4°C. However, when placed at 37°C, required to start the amplification phase of the assay, bacterial killing occurred immediately. This decline in numbers was followed by a logarithmic increase as the surviving bacteria multiplied. Both the extent of the initial decrease in numbers and the interval before logarithmic growth were directly related to the amount of CF added to the bacteria. The conclusions from these experiments were threefold: 1) as much as 25 μl of CF exhibits little or no killing activity at room temperature or below during the initial incubation. 2) This volume of CF rapidly kills most bacteria during incubation at 37°C. 3) Once CF has been allowed to interact with bacteria in these concentrations at room temperature, without lethal effect, it retains its ability to kill the bacteria when placed at 37°C, even though the reaction mixture has been diluted ninefold with TSB. This killing is not due simply to low levels of CF present after dilution with TSB because 25 μl CF are inactive if added to bacteria already diluted with TSB. Therefore, we must conclude that the CF and bacteria interact or bind during the initial incubation period at room temperature. We showed that this binding could be disrupted by the vigorous mixing in a very diluted medium which occurred during the course of the plating assay so that a spuriously low apparent activity was recorded. When TSB was added to the reaction mixture, GAF remained bound to the bacterium and was cidal at the optimal temperature.

The fact that bacteria/GAF interaction at room temperature could be reversed by vigorous agitation and dilution suggested that the active factor might be bound to the outer surface of the bacterium. We showed that free GAF could be readily inactivated by trypsin (Table II). In view of this, it was of interest to see whether we could demonstrate cell-bound GAF by its trypsin sensitivity. Indeed, the addition of trypsin to the putative bacteria-GAF complex progressively reduced bactericidal

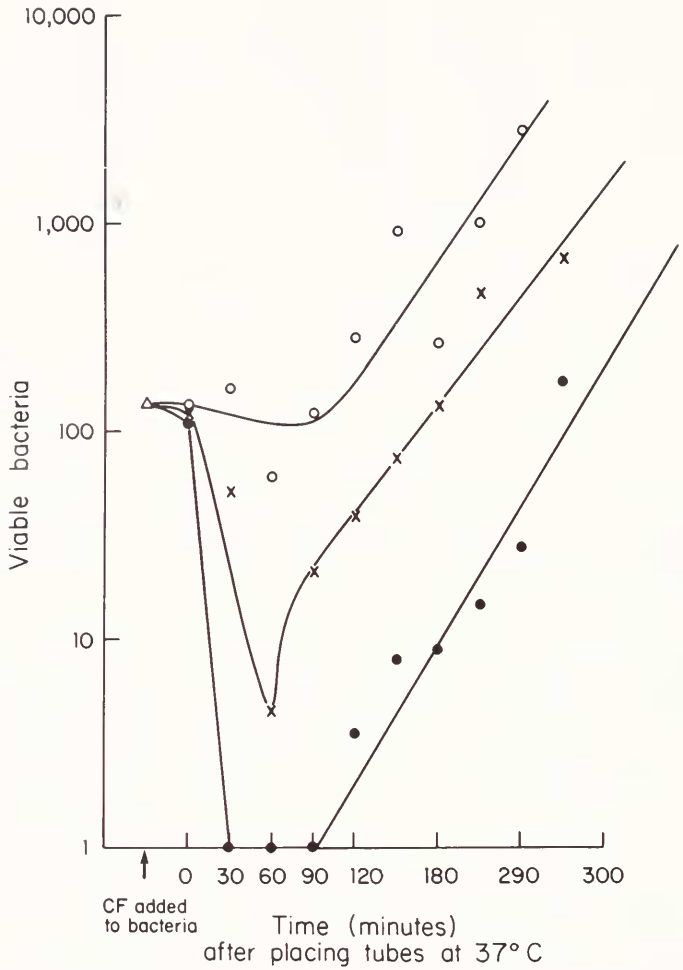

FIGURE 3. Analysis of turbidometric assay using plate counts. The bacteria and coelomic fluid (X = 10 μl CF; ● = 25 μl CF; ○ = no CF) were incubated together at room temperature for 30 min and 4 ml of tryptic soy broth was added to the mixture. A sample was taken from each tube, diluted (1:100) and 100 μl plated. All tubes were immediately placed at 37°C and sampled every 30 min and the number of viable bacteria determined. Points represent the average of duplicate plate counts; to obtain actual number of bacteria, multiply by 4.6×10^3.

activity. These results reinforce the idea that the factor binds initially to the outer surface of the bacterium.

Kinetics of the binding and killing reaction

Killing, as measured by the turbidometric assay, was shown above to consist of two parts: an initial incubation period during which bacteria and GAF associate in a way which can be reversed under certain conditions, and a subsequent period at 37°C (the amplification stage of the assay), during which the bacteria undergo GAF-mediated killing. Assuming that at short incubation periods the limiting factor in the killing reaction is the speed at which "killing units" of GAF attach to bacteria, the turbidometric assay gives a measure of the rate of binding. The kinetics of cell binding by GAF were extremely fast (Fig. 4a). The initial rate of reaction varies very

TABLE II

The trypsin sensitivity of GAF before and after it has bound to the bacterial surface

Treatment (length of incubation)	Effect of trypsin on bactericidal activity of GAF (% bacteria killed)	
	free GAF	bound GAF
No trypsin	100	100
Trypsin (15 min)	15	90
Trypsin (30 min)	0	64
Trypsin (60 min)	0	46

Bactericidal activity was measured by the turbidometric assay using 10 μl CF and a 15 min incubation period. Trypsin, at a final concentration of 200 μg/ml, was added before or after the bacteria/CF incubation as specified above. All trypsin incubations were carried out at room temperature. The presence of trypsin alone was shown not to affect bacterial growth.

sharply with concentration and cannot be accurately measured by this technique at CF concentrations >15 μl. A double logarithmic plot of rate versus concentration (Fig. 4b) has a slope between 3 and 4.

The initial rate of binding as a function of temperature is shown in Figure 5a; over the range 0–37°C an Arhenius plot of the rates (Fig. 5b) lies close to a straight line, with a low Q_{10} of about 1.6.

In contrast to the turbidometric assay, the results of the plate assay are a direct measure of the rate of the first irreversible step of the sequence of events leading to the death of the bacterium. At room temperature the rate of killing is rather slow and extremely variable. Therefore, the relation between rate of killing and concentration of GAF was investigated at 37°C (Figs. 6a, b). Unlike the rate of binding, the rate of reaction approaches a limiting value with increasing concentration. Figure 6c shows that the data, at least at concentrations greater than 5 μl, can be fitted by a hyperbolic function, with a V_{max} of approximately 5×10^5 bacteria/minute. The interpretation of these data will be discussed below, but the comparison of the kinetics of the initial binding to those of the killing reaction are striking. With 10 μl of CF the initial rate of binding (Fig. 4b) is 19.8×10^4 bacteria/minute, while the initial killing rate is 4.3×10^4 bacteria/minute (Fig. 6b). For 15 μl the corresponding figures are 105×10^4 and 6.8×10^4 bacteria/minute. It is apparent that for all but very low concentrations of CF, the bacteria bind GAF before there is any appreciable killing even at 37°C.

The temperature dependence of the killing reaction was difficult to measure accurately because of the variability of the data below 37°C (Table III). However, if one assumes a constant Q_{10} in the range 21–37°C, the data of Table III yield a value of Q_{10} of 2.5 ± 0.4 ($\bar{X} \pm$ S.D.), which is within the range of that found for many biochemical processes.

As has been explained above, since the killing reaction is allowed to go to completion at 37°C, the results of turbidometric assay are a measure of the total bactericidal capacity of a given volume of CF, provided sufficient time is given before the addition of tryptic soy broth for all available GAF to react with bacteria (greater than 15 minutes as shown in Fig. 4). Figure 7a shows the dose response obtained in this way at very low CF concentrations. The curve is sigmoidal, indicating either that the cooperative effect of more than one component of CF is required for bactericidal action, or that a multiple binding of one component is required to kill a bacterium. For multiple hit kinetics, the assumption of Poissonian probability distribution can be used to calculate theoretical dose-response curves (Mayer, 1961;

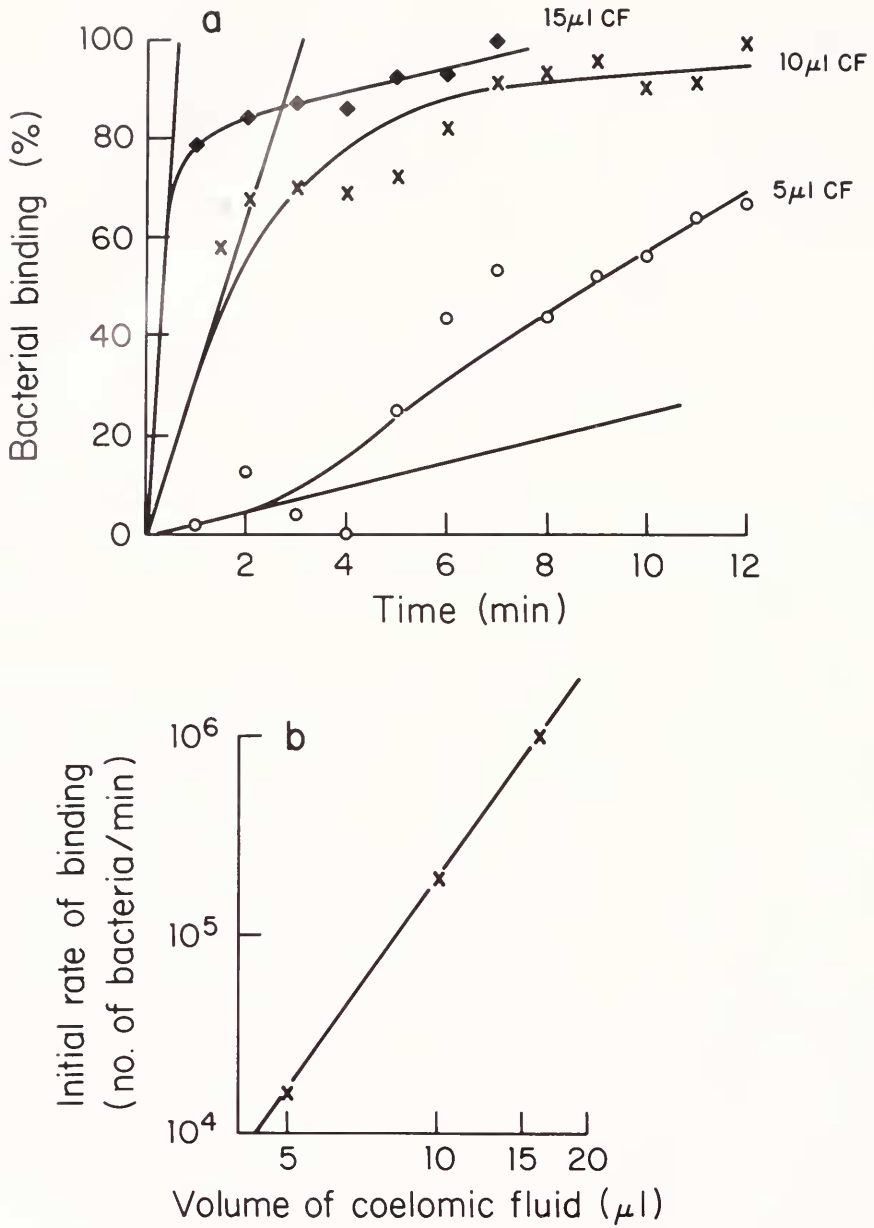

FIGURE 4. a) Rate of binding of GAF to bacteria using different CF volumes (O = 5 μl CF; X = 10 μl CF; ♦ = 15 μl CF). Bacteria were added to a given volume of coelomic fluid and allowed to react for 1–12 minutes. At the end of this period unbound coelomic fluid was diluted out with 4 ml tryptic soy broth and the bacteria grown up in the usual way. All bacteria which had bound sufficient GAF during the original incubation period were killed during the subsequent growth period. Bacterial killing was calculated directly from relative optical density as described in Methods.

b) Initial rates of binding (number of bacteria per min) were calculated from tangents drawn through the origin in Figure 4a. Initial rates are plotted as a function of concentration on a double-logarithmic plot. The slope of the straight line drawn through the three points is 3.8.

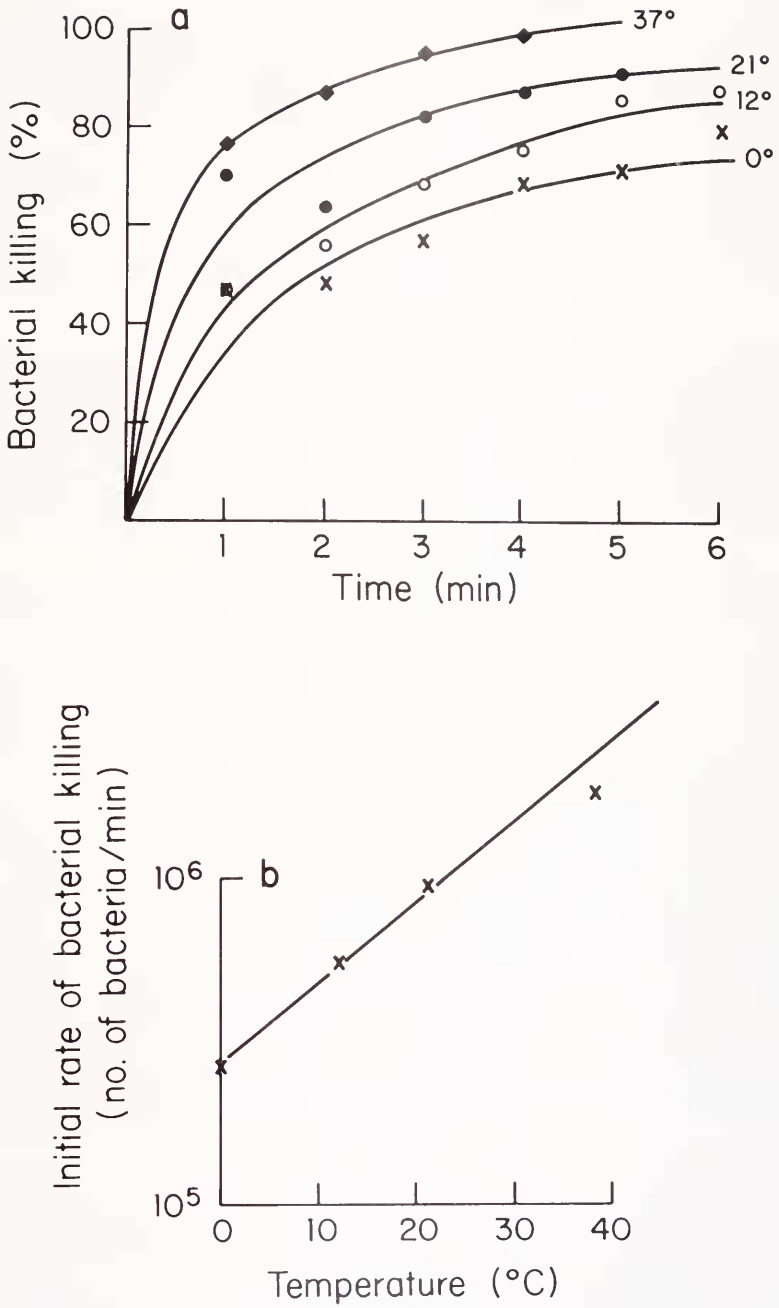

FIGURE 5. a) The rate of binding of GAF to bacteria at different temperatures (X = 0°, O = 12°, ● = 21°, ♦ = 37°C). The experiment was carried out as described in Figure 4; 10 μl of coelomic fluid was used in all cases. All determinations were done in triplicate and the average optical density was used to calculate bacterial killing.

b) An Arhenius plot of initial rate of bacterial killing as a function of temperature. The rates of killing were measured from the data shown in 5a, as described in Figure 4b.

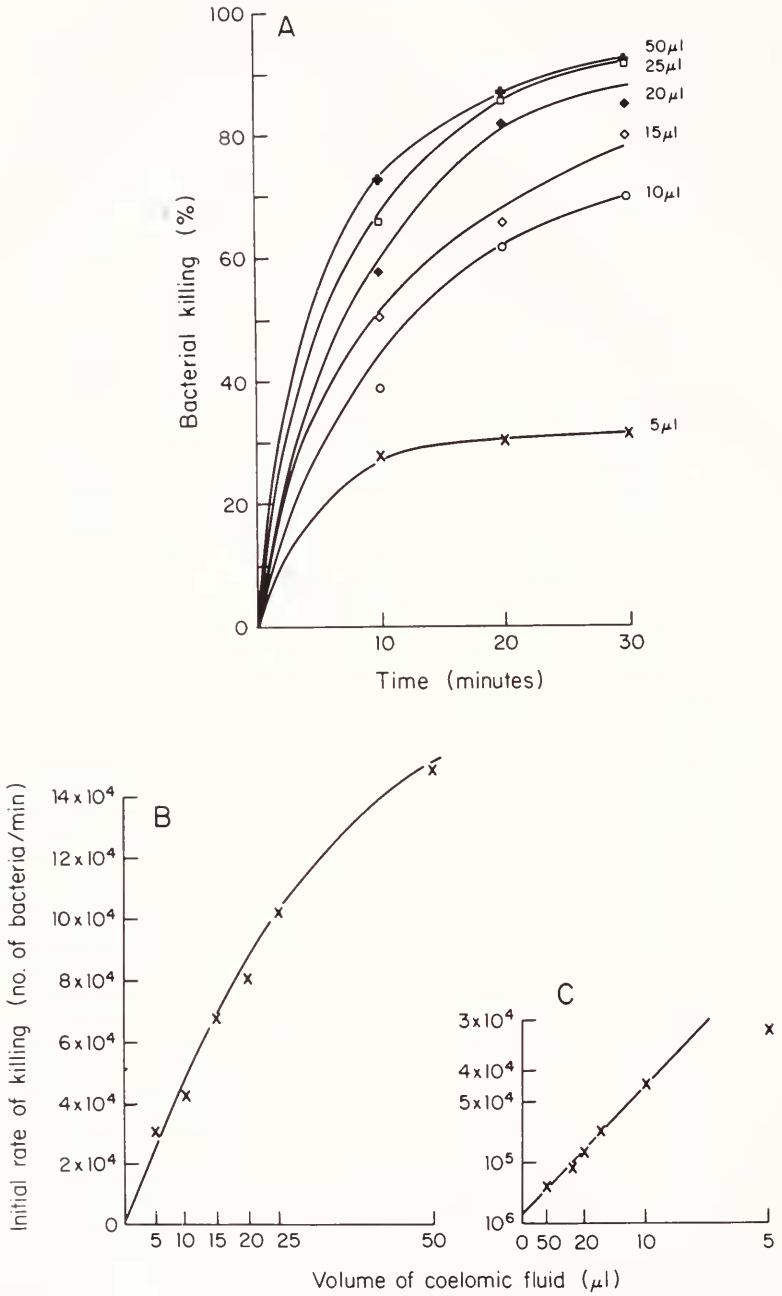

FIGURE 6. a) The rate of bacterial killing with different volumes of coelomic fluid. Bacteria and coelomic fluid were incubated together for the specified time and surviving organisms were counted by plating in tryptic soy agar as described in Methods. All values are the average of triplicate determinations.

b) Initial rate of killing as a function of coelomic fluid volume. The initial rates were obtained from the tangents (at 5 = 0) to the curves in 6a).

c) The data of 6b replotted in a double reciprocal form. The data approximate to a straight line at coelomic fluid concentrations greater than 5 μl. The intercept on the y axis, corresponding to the limiting velocity at high concentrations of coelomic fluid, is 5×10^5 bacteria/minute.

TABLE III

Temperature dependence of GAF activity

Temperature	Average initial rate of bacterial killing (bacteria/min)	Standard deviation (N)	Range
21°C	1.2×10^4	0.7×10^4 (4)	$0.6–2.6 \times 10^4$
37°C	5.1×10^4	0.9×10^4 (3)	$4.0–6.3 \times 10^4$

The initial rate of bacterial killing, as measured by the plate assay, was calculated as described for Figure 6.

Wright and Levine, 1981b). Such curves, plotted as the log of the proportion of surviving bacteria versus number of killing units/bacterium present for 1, 2, 3 or 4 hit models, are shown in Figure 7b. The curves in all cases approach a straight line at sufficiently high concentrations, and the intercept on the y-axis gives a measure of the multiplicity. A similar plot for the data in Figure 7a is shown in Figure 7c, and would indicate that 3 or 4 hits are required for the lethal action of GAF.

DISCUSSION

The principal conclusion of this paper is that the bactericidal action of GAF can be broken down experimentally into a two-step process of the type:

$$n(\text{GAF}) + \text{Bacterium} \xrightarrow{k_1} \text{Bacterium-GAF}_n \text{ complex} \xrightarrow{k_2} \text{Bacterium (killed)}$$

The evidence for this two-step model, involving an initial rapid, reversible binding reaction, followed by a slower killing reaction, and the methodology used to study the two processes have been presented in detail in the Results section.

The rate of binding of GAF to the outer surface of the bacterium is extremely rapid. The Q_{10} of this reaction (1.7) also indicates a physical binding type reaction, with a low activation energy, rather than a process involving metabolic reactions. Furthermore, GAF remains trypsin-sensitive when bound to the bacterium, indicating that at least some portion of the molecule is still exposed to the medium. The binding of GAF is, however, strongly influenced by the ionic composition of the medium. Not surprisingly, optimum binding occurs in ASW whose composition is very similar to *Glycera* CF.

Two separate pieces of evidence suggest that more than one molecule of bound GAF is required to kill a bacterium. From the law of mass action, the initial rate of binding ($V_{t=0}$) should be given by:

$$V_{t=0} = R_1(\text{GAF})^n[\text{bacteria}]$$

If the number of bacteria is kept constant, this can be rewritten as:

$$\text{Log } V_{t=0} = n \log [\text{GAF}] + \text{constant}$$

where n, the slope of this logarithmic relationship, is the number of molecules required for a lethal hit. A double-logarithmic plot of initial rate data was shown in Figure 4b and has a slope of 3.8, suggesting that between 3 and 4 molecules of GAF are required to kill a single bacterium.

An alternative approach to test the multiple-hit model is to allow the binding of free GAF to go to completion and look at the dose-response curve at low GAF levels (Fig. 7). As has been pointed out above, the theoretical dose-response curves

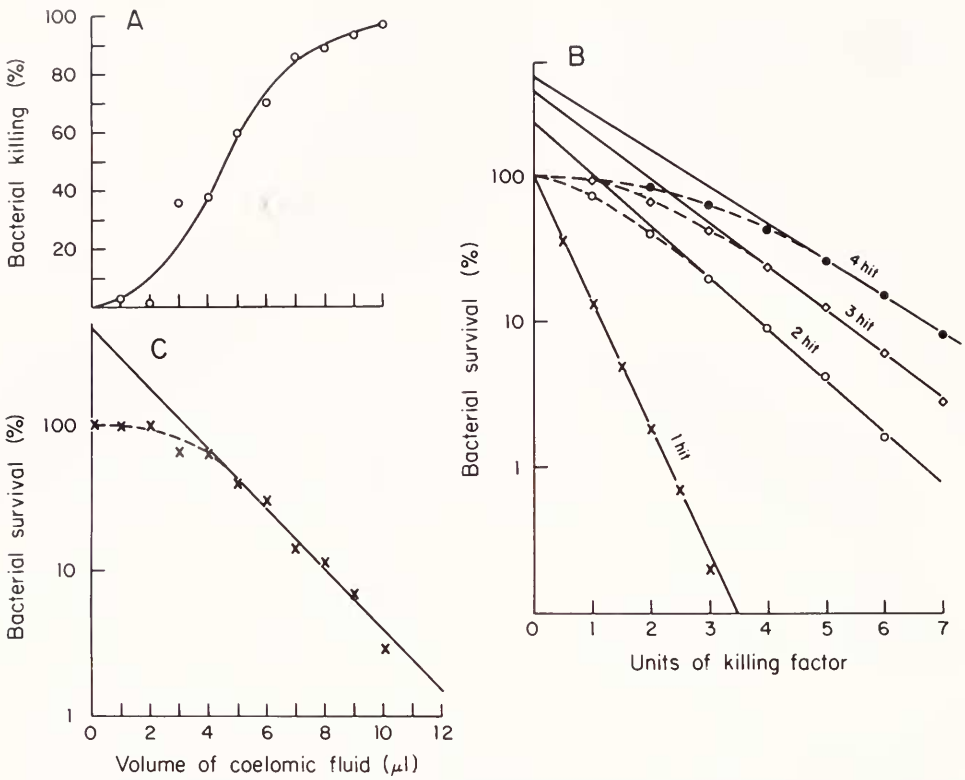

FIGURE 7. a) Dose-response curve at low volumes of coelomic fluid using the turbidometric assay. Bacteria and coelomic fluid were incubated together at room temperature for 30 min to allow complete binding of all free GAF before the addition of tryptic soy broth. Each point is the average of 5 determinations.

b) Theoretical curves of log (% survival) as a function of units of killing factor per bacterium for 1, 2, 3 and 4 hit models. Values are calculated from the Poisson distribution:

$$P_n = \left(1 + \lambda + \frac{\lambda^2}{2!} \cdots + \frac{\lambda^n}{n!}\right) e^{-\lambda}$$

where p is the probability of a bacterium binding n units of killing factor and λ is the total number of units present for each bacterium. $P_n - 1$ is, therefore, the probability of survival for a n hit model.

c) Plot of the data of 7a as log (% survival) as a function of coelomic fluid volume. The curve fits the multiple-hit models of 7b and the intercept on the y axis lies between that of a 3- and 4-hit model.

can be calculated for single and multiple hit models on the assumption that the chance of a molecule of GAF interacting with a bacterium is governed by Poissonian probability distribution. Such an approach has been used extensively in analyzing complement hemolytic (Mayer, 1961) and bactericidal action (Wright and Levine, 1981b). A comparison of theoretical-to-actual dose-response curves again indicates that a model requiring 3 or 4 hits/bacterium provides the best fit to the data (Figs. 7b, c). Since both initial binding rate data and dose-response curve data can theoretically be explained by a model requiring several different components of the CF to act cooperatively to kill a bacterium, this possibility cannot be conclusively dismissed.

The data discussed so far were all generated using the turbidometric assay of bacterial killing. In contrast, the plate assay gives a measure of the rate at which bacteria were actually killed (*i.e.*, the combined rate of both GAF binding and killing

reactions). Furthermore, since binding is relatively fast, the rate-limiting (and hence, rate-determining) step is the second one, and the plate assay actually measures the rate at which bound GAF causes an irreversible change in the bacterium leading to cell death. The initial rate increases with GAF concentration at low concentrations (Fig. 6b), but levels off at higher concentrations. This limiting velocity, presumably determined solely by the rate of the physiological reaction causing the death of the bacteria, can be calculated by plotting a double-inverse plot of the rate versus concentration (the Lineweaver/Burke plot of enzymologists), as shown in Figure 6c. The limiting velocity obtained by this method is approximately 5×10^5 bacteria/minute, or approximately 80% of the bacteria per minute.

The rate of killing continues to increase at concentrations several times larger than the minimum required to obtain 95% bacterial killing (Fig. 6a), implying that extra molecules of GAF speed up the rate at which a bacterium is killed.

Bacterial killing rate is considerably inhibited at room temperature versus 37°C. One possible explanation for this phenomenon is that the action of GAF requires some metabolic reaction to take place. Alternatively, GAF activity may be influenced by the physical state of the bacterial cell membrane, which is known to vary substantially at different temperatures (Stein *et al.*, 1969; Melchior *et al.*, 1970; Jacobson, 1976).

A third possibility, namely, that GAF action requires the bacteria to be actively dividing in order to be effective can be dismissed since bacteria are routinely killed while still in stationary phase (see, for example, Fig. 3). If the optimal killing temperature for GAF depends on properties of the target organism, such as membrane fluidity, it will be necessary to extend these experiments to strains of bacteria isolated from the immediate natural environment of *Glycera*. Perhaps GAF activity against such organisms would be optimal at temperatures more physiological for both polychaete and bacterium. Such experiments will also be of value in assessing more fully the role of GAF in the natural defense systems of *Glycera*.

It does not appear that GAF is identical to other antibacterial factors found in invertebrate sera, although few of these have been studied in sufficient detail to permit detailed comparison. GAF does not possess any of the properties of the lysozyme-type enzymes which have been described in a number of other bactericidal systems (Powning and Davidson, 1973; Cheng and Rodrick, 1974; Jollès and Jollès, 1975; Cheng *et al.*, 1977; Anderson and Cook, 1979; Cheng, 1980). Unlike lysozyme, GAF does not lyse *Micrococcus luteus* cell walls and is most active against Gram-negative bacteria (Anderson and Chain, 1982). It is also quite distinct from the inducible bacteriolytic proteins which have been recently isolated from the hemolymph of lepidopteran pupae (Hültmark *et al.*, 1980). There are some mechanistic similarities, however, between GAF and vertebrate complement. The antibacterial action of complement has recently been reexamined in detail (Wright and Levine, 1981a, b). Like GAF, complement action involves an initial binding reaction to the cell surface, followed by a killing reaction (Levine *et al.*, 1953; Muller-Eberhard, 1968); it has a low multiplicity, probably requiring two "killing units" per bacterium (Wright and Levine, 1981b); it is highly temperature-sensitive (Mayer and Levine, 1954), and has an absolute requirement for divalent cations (Levine *et al.*, 1953; Muschel and Treffers, 1956b). At the other end of the evolutionary spectrum, similar characteristics are shown by certain types of colicins (*e.g.*, K1 and E1), which are bactericidal proteins of bacterial origin (Weiss and Luria, 1978; Wendt, 1970). It is extremely unlikely that there is any true homology between GAF and either complement or the colicins. Rather, the similarities probably arise from parallel strategies evolved in these diverse organisms to eliminate competing or pathogenic microorganisms.

ACKNOWLEDGMENTS

This work was supported by Grants OCE-7723443 and OCE-8016186 from the National Science Foundation; Grant CA-08748 from the National Cancer Institute, and a Grant from the Griffis Foundation.

Dr. Chain is a Harkness Fellow of the Commonwealth Fund of New York.

LITERATURE CITED

ANDERSON, R. S., AND B. M. CHAIN. 1982. Antibacterial activity in the coelomic fluid of a marine annelid, *Glycera dibranchiata*. *J. Invertebr. Pathol.* **40:** 320–326.

ANDERSON, R. S., AND M. L. COOK. 1979. Induction of lysozyme activity in the hemolymph and hemocytes of an insect, *Spodoptera eridania*. *J. Invertebr. Pathol.* **33:** 197–203.

CHENG, T. 1980. Non-hemocyte sources of certain lysozomal enzymes in *Biomphalaria glabrata* (Mollusca: Pulmonata). *J. Invertebr. Pathol.* **35:** 107–108.

CHENG, T. C., AND G. E. RODRICK. 1974. Identification and characterization of lysozymes from the hemolymph of the soft shelled clam, *Mya arenaria*. *Biol. Bull.* **147:** 311–320.

CHENG, T. C., M. J. CHORNEY, AND T. P. YOSHINO. 1977. Lysozyme-like activity in the hemolymph of *Biomphalaria glabrata* challenged with bacteria. *J. Invertebr. Pathol.* **29:** 170–174.

GUIRARD, B. M., AND E. E. SNELL. 1962. Nutritional requirements of microorganisms. Pp. 36–38 in *The Bacteria* VI. I. C. Grunsalus and R. Y. Stanier, eds., Academic Press, New York and London.

HÜLTMARK, O., H. STEINER, T. RASMUSSON, AND H. G. BOMAN. 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.* **106:** 7–16.

JACOBSON, M. B. 1976. Differential scanning calorimetry of *E. coli* lipids and membranes. *Fed. Proc.* **35:** 1531.

JOLLÈS, J., AND P. JOLLÈS. 1975. The lysozyme from *Asterias rubens*. *Eur. J. Biochem.* **59:** 19–23.

LEVINE, L., A. G. OSLER, AND M. M. MAYER. 1953. Studies on the role of calcium and magnesium in complement fixation and immune hemolysis. III. The respective roles of calcium and magnesium in immune hemolysis. *J. Immunol.* **71:** 374–379.

MAYER, M. M. 1961. Development of the one-hit theory of immune hemolysis. Pp. 268–279 in *Immunochemical Approaches to Problems in Microbiology*, M. Heidelberger and O. J. Plescia, eds. Institute of Microbiology, Rutgers, The State University.

MAYER, M. M., AND P. LEVINE. 1954. Kinetic studies on immune hemolysis. IV. Rate determination of magnesium and terminal reaction steps. *J. Immunol.* **72:** 516–530.

MELCHIOR, D. L., H. J. MOROWITZ, J. M. STURTEVANT, AND T. Y. TSONG. 1970. Characterization of the plasma membrane of *Mycoplasma laidlawii*. VI. Phase transitions of membrane lipids. *Biochim. Biophys. Acta* **29:** 119–122.

MÜLLER-EBERHARD, H. J. 1968. The chemistry and reaction mechanisms of complement. *Adv. Immunol.* **8:** 1–80.

MUSCHEL, L. H., AND H. P. TREFFERS. 1956a. Quantitative studies on the bactericidal actions of serum and complement. I. A rapid photometric growth assay for bactericidal activity. *J. Immunol.* **76:** 1–10.

MUSCHEL, L. H., AND H. P. TREFFERS. 1956b. Quantitative studies on the bactericidal actions of serum and complement. II. Some implications for the mechanism of the bactericidal reaction. *J. Immunol.* **76:** 11–19.

POWNING, H. F., AND W. J. DAVIDSON. 1973. Studies on insect bacteriolytic enzymes. I. Lysozyme in the hemolymph of *Galleria mellonella* and *Bombyx mori*. *Comp. Biochem. Physiol.* **45B:** 669–686.

STEIN, J. M., M. E. TOURTELLOTTE, J. C. REINERT, R. N. MCELHANEY, AND R. L. RADER. 1969. Calorimetric evidence for the liquid-crystalline state of lipids in a biomembrane. *Proc. Natl. Acad. Sci. USA* **63:** 104–109.

WEISS, M. J., AND S. E. LURIA. 1978. Reduction of membrane potential, an immediate effect of colicin K. *Proc. Natl. Acad. Sci. USA* **75:** 2483–2487.

WENDT, L. 1970. Events in colicin K action. *J. Bacteriol.* **104:** 1236–1241.

WRIGHT, S. D., AND R. P. LEVINE. 1981a. How complement kills *E. coli*: I. Location of the lethal lesion. *J. Immunol.* **127:** 1146–1151.

WRIGHT, S. D., AND R. P. LEVINE. 1981b. How complement kills *E. coli*: II. The apparent two-hit nature of the lethal event. *J. Immunol.* **127:** 1152–1156.